REMARKS

Applicants submit this response in reply to the Office Action mailed August 24, 2006. In the Office Action, the Examiner has:

(1) objected to the title as not being descriptive;

(2) objected to claim 52 for certain informalities;

(3) rejected claims 52 and 60 under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification in such a way as to reasonably convey to a person that the inventor had possession of the claimed invention at the time of filing; and

(4) rejected claims 52-53, 55, 57, and 60-61 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention.

In connection with the present Response, claims 1-51, 54, 56, and 58-59 were previously cancelled, claim 57 is now being cancelled, and claims 52 and 60 have been amended. No new subject matter has been added in connection with the amendments. Claims 52-53, 55 and 60-61 remain pending in the present application.

In view of the above changes and the following remarks, the Applicants respectfully request reconsideration of the claims.

Title

With respect to item (1), the title of the present application has been amended to clearly indicate the invention to which the claims are directed. The amended title now reads "Systemic Antibody Production by Genetically Modified Cells in Mammals."

Informalities

With respect to item (2), claims 52 has been amended to address the informalities noted by the Examiner. Claim 57, on the other hand, has been cancelled.

35 U.S.C. § 112

With respect to item (3), the Examiner has stated that new matter had been introduced in the claims 52 and 60 in the response to the previous Office Action. The Applicants respectfully disagree with this assertion by the Examiner.

Indeed, in claim 52, support for the recitation "without triggering an anti-idiotype response directed against said antibody in said mammal" can be found in paragraph 97 of the

specification. Specifically, paragraph 97 recites "No anti-idiotype response could be detected under these conditions." In other words, no anti-idiotype response could be detected by ELISA.

The Examiner has stated that the disclosure of the instant Patent Application fails to address any issue regarding the prevention of an anti-idiotypic response against the recombinant antibody.

If the Examiner may note, the present invention comes from the observation that, in a subject mammal transplanted with non-B cells derived from the subject mammal or from another compatible donor mammal that have been genetically modified to produce and secrete antibodies, the produced antibodies show biophysical binding properties (k_{on} and k_{off} and K_A et K_D) comparable to those of antibodies naturally produced by cells of the B lineage. In addition, these recombinant antibodies were shown not to trigger any anti-idiotypic response.

Before the experimental work presented in the present application, one could not state that no adverse anti-idiotypic response would be elicited against antibodies produced by genetically modified non-B cells. In fact, there were two major causes of concern for one skilled in the art at that time. First, the possibility of differences in post-translational modifications, such as glycosylations, between antibodies naturally produced by B-cell type cells and recombinant antibodies produced by genetically modified non-B cells had to be considered, as this might have conferred antigenic properties to the latter antibody. Moreover, recombinant antibodies produced by genetically modified cells would be produced in a tissue environment, such as muscle, skin or liver, which differ from the natural environment of antibody-secreting plasmocytes. Therefore, it could not be excluded that this new environment, due to its particular composition, would play an adjuvant role favoring the induction of an anti-idiotypic response against the recombinant antibodies. Moreover, this could have potentially been aggravated by possible differences in post-translational modifications with antibodies naturally produced by plasmocytes.

To further support the observed absence of adverse anti-idiotypic responses against recombinant antibodies produced in vivo by cells not naturally producing antibodies, the Applicants respectfully submit herewith the publication by Noël and Piechazyk et al. (Journal of Investigative Dermatology (2000) 115, 740-745). In the work reported in this publication, the authors have shown that, in immunocompetent mouse transplanted with fibroblastic cells expressing antibodies, no anti-idiotypic response could be detected. The present invention establishes for the first time that no specific procedure is required to avoid the induction of an anti-idiotypic response against an ectopic antibody produced in a mammal upon genetic

modification of cells that do not naturally produce antibodies. This was confirmed in the above-mentionned publication by Noël et al.. In this sense, the disclosure provides the general guidance for producing an antibody in a subject as it indicates that standard expression systems for producing an antibody are sufficient for not inducing any adverse anti-idiotypic response in the treated subject.

The Examiner also states that the U.S. Patent No. 6,426,088 provides examples where certain levels of Tg10 in mice would produce an anti-idiotype response.

The Applicants respectfully note that the experiments disclosed in U.S. Patent No. 6,426,088, which were performed by the inventors of the present invention, were performed with the Tg10 antibody in the presence of Freund's adjuvant. This procedure was used, in experiments paralleling to those of implantation of recombinant antibody-producing cells not inducing any anti-idiotypic responses, as a positive control to show that the mice used in the trial were not intrinsically refractory to the induction of an anti-idiotype response. In this case, the response could be initiated due to the pro-inflammatory effect of the Freund's adjuvant.

Turning to claim 60, if the Examiner may note, the recitation "production of antibodies displaying thermodynamic and kinetic properties similar to those initially produced by B-cell lineage cells" is supported by paragraphs 70, 71 and Table 1 of the specification. Table 1 shows the association- (k_{on}) and dissociation (k_{off}) constants as well as the affinity constant (K_A) of a recombinant antibody produced according to the present invention towards its cognate antigen. The former two constants (k_{on} and k_{off}) define the kinetic properties of recognition of the cognate antigen by the antibody and the latter constant (K_A), the thermodynamic property of binding of the antibody to its cognate antigen that is often refered as the stability factor of the interaction. The results obtained with recombinant and natural antibodies do teach that k_{on}, k_{off} and K_A for both type of antibodies are similar.

The Examiner states that there is not general guidance for how thermodynamic and kinetic properties are assayed. The Applicants respectfully disagree.

The specification includes, by reference, the publication of Fagerstam et al., which details the use of Biosensor techniques (paragraph 70) and specifies that k_{on} and k_{off} can be measured by Surface Plasmon Resonance using the BiaCore technology. The BiaCore technology is currently the most commonly used and most powerful technique to measure association- and dissociation kinetic constants and deduce the affinity constant, as the latter is the ratio between the former two values. Taking into account the teaching of Fagerstam et al. and the BiaCore technology and using his general knowledge in immunology, the Person

Skilled in the Art has sufficient guidance for how thermodynamic and kinetic properties are assayed. Others techniques may also be used to measure these antibody features as well and are also part of the general knowledge.

Furthermore, the Examiner states that the working example only provides for measurement by surface plasma resonance, not any thermodynamic or kinetic property. The Applicants respectfully disagree.

Indeed, as mentioned above, the kinetics of association and dissociation, which are described by the k_{on} and k_{off} constants, respectively, are clearly disclosed. The thermodynamic constant of association, K_A can be calculated from the kon and koff values by applying the formula $K_A = k_{on} / k_{off}$. In addition, the constant of dissociation, K_D, can readily be determined by applying the relation $K_D = 1/K_A$. It is to note that these formulas are well known by the person skilled in the art and can be found in any chemistry or biochemistry book. Accordingly, the instant application contains all the essential teaching to allow the person skilled in the art to reproduce the claimed method.

With respect to item (4), the comments provided above are repeated. In addition, claims 52 and 60 have been amended in the manner noted above.

With respect to the statement by the Examiner that claims 60 and 61 fail to limit claim 52 because these claims just specify inherent characteristics of the antibody produced.

The Applicants respectfully note that such limitations are permitted if they do not broaden the scope of the claim 52. Generally, in a dependent claim, it is not forbidden to specify characteristics that are inherent to the invention.

In light of the foregoing, the Applicants respectfully request withdrawal of the 35 U.S.C. 112 rejections.

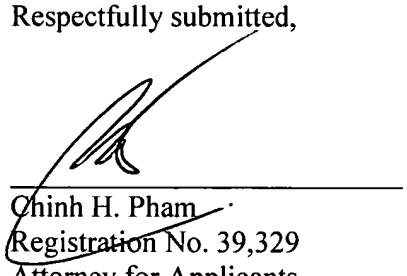
Conclusion

In view of the foregoing remarks, Applicants submit that the pending claims clearly and distinctly set forth the subject matter of the present invention.

Accordingly, Applicants submit that the claims are now in condition for allowance. Withdrawal of the pending rejections, and early and favorable reconsideration are respectfully solicited. In the event that a telephone conversation would further prosecute and/or expedite allowance, the Examiner is invited to contact the undersigned at (617) 310-6000.

Applicants further request a two (2) month extension of time in connection with the filing of this Response and authorize the Examiner to charge an amount of $225 to Deposit Account No. 50-2678 to cover the extension fee. Applicants do not believe that any extension or additional fee is required in connection with this Response However, should any extension or fee be required for timely consideration of the present application, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this application be charged to Deposit Account No. 502775.

Respectfully submitted,



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Sustained Systemic Delivery of Monoclonal Antibodies by Genetically Modified Skin Fibroblasts

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In vivo* production and systemic delivery of therapeutic antibodies by engineered cells might advantageously replace injection of purified antibodies for treating a variety of life-threatening diseases, including cancer, acquired immunedeficiency syndrome, and autoimmune diseases. We report here that skin fibroblasts retrovirally transduced to express immunoglobulin genes can be used for sustained long-term systemic delivery of cloned antibodies in immunocompetent mice. Importantly, no anti-idiotypic response against the ectopically expressed model antibody used in this study was observed. This supports the notion that skin fibroblasts can potentially be used in antibody-based gene/cell therapy protocols without inducing any adverse immune response in treated individuals.** ***Key words: gene therapy/immunotherapy/monoclonal antibody/skin fibroblasts. J Invest Dermatol 115:000–000, 2000

Since their discovery, monoclonal antibodies (MoAb) have been widely used in basic and applied science as well as in diagnosis and therapy. In the latter case, they have mostly been used in short-term (several days to several weeks) clinical settings where some of them have proved particularly efficient, such as the IDEC-C2B8 (Rituximab) anti-CD20 MoAb that was shown to be more efficient than conventional chemotherapy for treating non-Hodgkin's lymphoma patients (Maloney *et al*, 1997); however, long-term administration of therapeutic MoAb might also be highly beneficial for treating many other diseases. An obvious first application is cancer therapy where passive immunosurveillance could potentially be used to prevent relapse after a primary surgical, radiotherapy, or chemotherapy treatment, when MoAb specific for tumor cells are available (Vitetta and Uhr, 1994; Scott and Welt, 1997). Other applications might include the treatment of severe viral diseases such as acquired immunodeficiency syndrome, possibly in combination with drug multitherapy, for neutralizing human immunodeficiency virus or killing virus-producing cells (Burton and Montefiori, 1997) as well as that of certain autoimmune diseases such as rheumatoid arthritis. In the latter case, it is worth mentioning phase III clinical trials where anti-tumor necrosis factor-α MoAb were shown to be efficient for the reduction of inflammation and the protection of cartilage and joints of patients (Maini *et al*, 1995). Long-term MoAb-based treatments, however, have encountered a number of hurdles and limitations. These include: (i) the restrictive cost of antibodies produced and certified for human use; (ii) the high frequency of MoAb injections because of the limited life spans of human immunoglobulins (half-lives range from 2.5 to 23 d depending on the isotype) (Carayannopoulos and Capra, 1993); (iii) the mild to severe adverse effects often associated with hour-long intravenous infusion that range from nausea, migraine, and diarrhea to anemia, neutropenia and even myocardis infraction (Maloney *et al*, 1997); and (iv) sometimes, the mounting of anti-idiotypic responses against the therapeutic MoAb, most likely in response to repeated massive delivery of MoAb (Isaacs, 1990; Kuus-Reichel *et al*, 1994).

To render long-term antibody-based therapy cost-effective, as well as to develop a single-step year-long treatment allowing continuous and sustained delivery of MoAb at low, but therapeutic, levels preventing the induction of neutralizing responses, we have explored the possibility of long-term *in vivo* production and systemic delivery of MoAb by genetically engineered cells (Noël *et al*, 1997; Pelegrin *et al*, 1998a,b). Plasmocytes, the cells naturally specialized in antibody production, cannot be used in such gene/cell therapy protocols because they are short-lived with a life span of a few days *in vivo* and already secrete an antibody. As a first step in our investigations, we demonstrated that various cell types amenable to gene therapy protocols can, upon genetic modification, produce MoAb *in vitro* and, subsequently, that long-term *in vivo* production and systemic delivery of an ectopic MoAb can be achieved in immunocompetent mice upon grafting of genetically modified myoblasts (Noël *et al*, 1997). With respect to the development of this technology, an important issue is now the identification of other cell types allowing *in vivo* production and systemic delivery of MoAb.

Skin fibroblasts are good candidates for this purpose because: (i) they can easily be expanded from skin biopsies; (ii) they can be genetically modified using retroviral vectors; (iii) they have been shown capable of producing MoAb *in vitro* upon antibody gene transfer (Noël *et al*, 1997); and (iv) they have already been successfully used to deliver varied proteins systemically, such as α-L-iduronidase (Salvetti *et al*, 1995), β-glucoronidase (Moullier *et al*, 1993a,b), and erythropoietin (Naffakh *et al*, 1995) for at least several months in animals such as mice and dogs where either correction of genetic deficiencies or modification of hematocrits were obtained. In these experiments, genetically modified skin

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Abbreviations: MuLV, murine leukemia virus; MoMuLV, Moloney murine leukemia virus; PGK, phosphoglycerate kinase; LTR, long tandem repeat.

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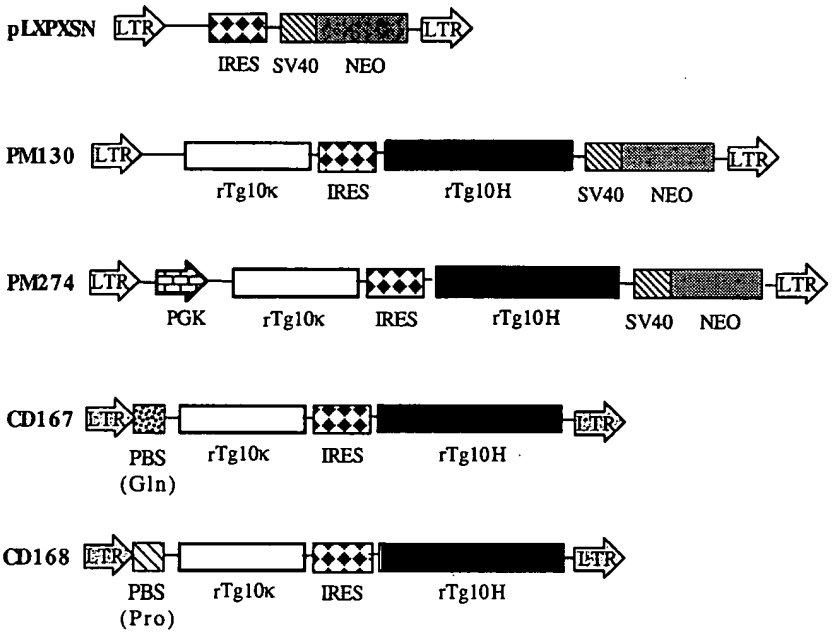

Figure 1. Tg10 MoAb-expressing retroviral vectors. Tg10 MoAb-expressing PM130 and PM274 vectors were derived from the pLXPXSN MoMuLV-derived retroviral vector. The latter contains the poliovirus internal ribosome entry site sequence allowing reinitiation of translation and expresses the neomycin gene resistance (neo) from an internal SV40 virus promoter (SV40). The light (rTg10κ) and heavy (rTg10H) chains of Tg10 were cloned upstream and downstream of the poliovirus internal ribosome entry site sequence, respectively. In the case of PM274 the phosphoglycerate promoter (PGK) was cloned upstream of the rTG10 light chain cDNA. To construct CD167 and CD168, a cassette bearing the Tg10 light and heavy chain cDNAs separated by the poliovirus internal ribosome entry site was PCR-amplified from PM130 and cloned into the Akv-derived ptvAkv-tRNA^{Pro} and ptvAkv-tRNA^{Gln-1} vectors which differ only at the level of their tRNA primer binding sites (see text and *Materials and Methods*).

fibroblasts were embedded into collagen lattices built upon synthetic polytetrafluoroethylene (PTFE) fibers before intraperitoneal implantation in animals where they formed stable vascularized neo-organs. Using the same approach, which permits an efficient cell grafting not achievable by simple injection whatever the location of the latter, we report that retrovirally transduced skin fibroblasts can be used for sustained systemic delivery of MoAb in immunocompetent mice. Importantly, no anti-idiotypic response against the model antibody used in this study could be detected in treated animals. Our data, thus, lend support to the notion that skin fibroblasts can potentially be used in antibody-based gene/cell therapy protocols without inducing any adverse immune response in treated individuals.

MATERIALS AND METHODS

Construction of retroviral vectors The PM130 vector (Noël *et al*, 1996) was generated by cloning the light (κ) and heavy (H) chain cDNA of the Tg10 MoAb into the Moloney murine leukemia virus (MoMuLV)-derived pLXPXSN retroviral vector (Morgan *et al*, 1992) (**Fig 1**). PM274 was constructed by cloning a 525 bp polymerase chain reaction (PCR)-amplified sequence carrying the phosphoglycerate kinase (PGK) promoter into the *Eco*RI site located upstream of the Tg10 κ light chain in PM130 (**Fig 1**) PCR amplification was carried out using the M48 retroviral vector (Moullier *et al*, 1995) and primers hybridizing upstream (5′GGG-GAATTCTACCGGGTAGGGGAGGCGCTT) and downstream (5′GGG-GAATTCGGATCCGGTCGAAAGCCC-GG) of the transcription initiation site. PCR products were subcloned into the pGEM-T vector (Promega) and recloned into the *Eco*RI site of PM130 using the *Eco*RI sites contained in amplification primers (underlined nucleotides). Plasmids CD167 and CD168 were derived from the Akv murine leukemia virus (MuLV)-based retroviral vectors ptvAkv-tRNA^{Gln-1} and ptvAkv-tRNA^{Pro} that harbor mutated and wild-type primer-binding sites matching tRNA^{Gln-1} and tRNA^{Pro}, respectively (Lund *et al*, 1993). A 3070 nucleotide fragment containing the κ and H chains of PM130 separated by the poliovirus internal ribosome entry site sequence was PCR-amplified using specific primers (5′GGGGGT-GATCAATGAAGTTGCCTGGTAGGC-TGTTG and 5′GGGGCT-TAAGCTATTTACCAGGAGAGTGGGAG-AGG-CTC and blunt end—cloned in the unique cloning restriction sites of ptvAkv-tRNA^{Pro} and ptvAkv-tRNA^{Gln-1} to give CD167 and CD168, respectively (**Fig 1**).

Retrovirus-producing cells PA317 (Miller and Buttimore, 1986) and FlyA (Cosset *et al*, 1995) are amphotropic packaging cell lines. They were cultured in Dulbecco's minimal essential medium (DMEM) (Gibco-BRL) containing 10% fetal bovine serum (Gibco-BRL), 100 μg streptomycin per ml, 100 U penicillin per ml, and 2 mM L-glutamine. Packaging cells were transfected with retroviral vector plasmids using the calcium phosphate precipitation method (Sambrook *et al*, 1989) as described in Noël *et al* (1997). Forty-eight hours after transcription, 1.2 mg per ml (FlyA) or 0.5 mg per ml (PA317) G418 (Gibco-BRL) was added to the culture medium that was changed every 3-4 d until individual clones were visible and, thereafter, expanded separately. To identify good retrovirus producer lines, 10–20 clones per transcription were tested for the production of the Tg10 MoAb. When a neomycin resistance gene was present in the vector (see **Fig 1**), viral particle production in culture supernatants was directly assayed. In this case, serial dilutions of retrovirus-containing culture supernatants were added for 16 h to NIH 3T3 cells plated in 12 well culture plates in the presence of 8 μg polybrene per ml (Sigma, St Louis, MO) and, after 10–12 d of culture in the presence of 0.6 mg G418 per ml, the number of resistant clones was scored. When no resistance gene was carried by the vector, retrovirus titers were estimated by comparing Tg10 MoAb production in culture supernatants with that of various PM130-producer cell lines producing known titers of retrovirus. This was possible because of the linear relationship existing between retrovirus production and Tg10 MoAb secretion under our experimental conditions (data not shown). PA96.7 and PA130.2 cells were derived from PA317 packaging cells and produce CD96 (10^5 cfu per ml) and PM130 (10^4 cfu per ml) retroviral vectors, respectively (Noël *et al*, 1997), whereas FlyA167 (10^4 cfu per ml), FlyA168 (10^5 cfu per ml), and FlyA274 (10^5 cfu per ml) clones were derived from FlyA cells and produce CD167, CD168, and PM274 vectors, respectively.

Primary cell culture and gene transfer Primary fibroblasts were prepared from 4 d old C3H mice (IFFA CREDO, l'Arbresle, France) skin biopsies as previously described (Naffakh *et al*, 1995; Noël *et al*, 1997). Briefly, skin biopsies were digested in an enzymatic mixture containing 0.2% collagenase (Worthington Biochemical Corporation, Lakewood, NJ) and 20% dispase (Becton Dickinson, Bedford, MA) in DMEM for 2 h at 37°C. Primary fibroblasts released into the suspension were centrifuged at low speed (500× *g* for 5 min), rinsed four times in DMEM and resuspended in fresh DMEM containing 10% fetal bovine serum, 100 μg of streptomycin per ml, 100 units penicillin per ml, and 2 mM L-glutamine.

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Primary cells were plated at the density of 5×10^6 cells per 140 mm diameter petri dish and infected, twice a day for 4–5 d using 12 ml of culture supernatant from retrovirus-producing cells grown to confluence in the presence of 8 µg polybrene per ml for 2 h at 37°C. Retrovirus-containing cell culture supernatants were replaced by fresh DMEM at the end of infections. Cells were grown to confluence before being harvested for neo-organ preparation.

Neo-organ formation and implantation Expanded PTFE fibers (W. L. Gore and Associates, Flagstaff, AZ) were coated with rat tail collage type I (Sigma) and recombinant human basic fibroblast growth factor (Promega, Madison, WI) as described previously (Moullier *et al*, 1993a; Naffakh *et al*, 1995). Genetically modified fibroblasts were trypsinized, washed with phosphate-buffered saline (0.15 M NaCl, 0.01 M Na phosphate, pH 7) and 5×10^6 to 10^7 cells were resuspended in 2 ml of 10-fold concentrated RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 3 mg rat tail collagen type I and 20 ng basic fibroblast growth factor. This cell suspension was then layered on to the coated PTFE fibers placed on the bottom of a well of a 12 well culture plate (Corning) and cultured for 4 d; this time was necessary for allowing the packing of fibroblasts around PTFE fibers and contraction of the lattices. Two condensed lattices containing 10^7 cells were implanted into the peritoneal cavity of 4–6 wk old female C3H mice previously anesthetized using 0.01 ml per g of body weight of a solution containing 0.1% xylazine (Rompun, Bayer, Leverkusen, Germany) and 10 mg ketamine per ml (Imalgène, Rhône Mérieux, Lyon, France).

Immunoassays Tg10 MoAb contained in culture supernatants and sera was assayed by enzyme-linked immunosorbent assay (ELISA) using human thyroglobulin (Biogenesis, Poole, UK) for coating plates as previously described (Piechaczyk *et al*, 1985). Affinity chromatography-purified Tg10 antibody was used as a standard for quantification (Piechaczyk *et al*, 1985; Noël *et al*, 1997). Tg10 MoAb-containing lattice culture supernatants were concentrated 13-fold using Macrosep™ 10K centrifugal concentrators (Filtron Technology Corporation, Northborough, MA) by centrifugation at $5000 \times g$ at 4°C for 3 h 30 before the ELISA. Anti-idiotypic antibodies directed against the Tg10 antibodies in the mice sera were assayed by ELISA using Tg10 $F(ab')_2$ fragments for coating plates and rabbit (10 µg per ml) and mouse (1/125 dilution) anti-idiotypic antisera against the Tg10 antibody as positive controls (Del Rio *et al*, 1995; Pelegrin *et al*, 1998a). The immunization of mice with the Tg10 MoAb is described in Pelegrin *et al* (1998a). The specificity of the anti-idiotypic response was further assayed using both a competition and inhibition assay. In the former test, peroxidase-conjugated Tg10 antibody was added to mouse sera for an overnight incubation at 4°C before transfer of the mixtures to wells of human thyroglobulin-coated ELISA plates. In the latter, mice sera were added to Tg10 $F(ab')_2$ fragment-coated ELISA plates before the addition of a rabbit anti-Tg10 idiotype anti-serum conjugated to horseradish peroxidase (Del Rio *et al*, 1995; Pelegrin *et al*, 1998a).

RESULTS AND DISCUSSION

Retroviral vectors for the production of Tg10 MoAb by genetically modified skin fibroblasts Tg10 is a IgG2a/κ mouse MoAb directed against human thyroglobulin (Piechaczyk *et al*, 1985). It was chosen here as a model antibody for two reasons. First, a highly specific and sensitive ELISA for it is available (Piechaczyk *et al*, 1985) and, thus, permits detection of low concentrations in blood samples. Second, it can trigger an anti-idiotypic response against it when injected into mice under immunogenic conditions (Del Rio *et al*, 1995). As one of the major limitations of repeated MoAb infusion is the possible development of neutralizing anti-idiotypic responses by treated individuals, this criteria has to be met for testing whether skin fibroblast-mediated delivery of MoAb can also stimulate an anti-idiotypic response in immunocompetent grafted mice. It is also worth mentioning that the half-life of Tg10 in the mouse is 4–5 d (Pelegrin *et al*, 1998a) (as that of other IgG2a immunoglobulins, Vieira and Rajewsky, 1988; Israel *et al*, 1996), and that this short half-life allows rapid visualization of any decrease in Tg10 production *in vivo* in case of gene expression shut off or alteration of grafts.

Expression of transduced genes can vary according to the retroviral vector used and, sometimes, be more or less rapidly shut off *in vivo*. This is particularly true in the case of long tandem repeat (LTR) – driven transcription as in vectors derived from MoMuLV, the parental virus for most retroviral vectors used so far (Karavanas *et al*, 1998). To circumvent this possible drawback, four different MuLV-derived transcriptions (see **Fig 1** and *Materials and Methods*) were constructed to maximize the chance of long-term expression upon Tg10 gene transfer into skin fibroblasts because transcription driven by MoMuLV. In all vectors, Tg10 heavy and light chain cDNA were translated from a monocistronic RNA owing to the poliovirus internal ribosome entry site. Transcription was under the control of the retroviral LTR in three vectors (PM130, CD167, and CD168) and, in once case (PM274), from an internal

Table I. *In vitro* production of Tg10 MoAb by engineered skin fibroblasts

Vector	Tg10 antibody in culture supernatants of genetically modified primary skin fibroblasts (ng per 10^7 cells per 48 h: mean ± SD)		Reduction factor
	Cells in monolayer	Cells in lattice	
pLXPXSN	0	0	0
PM130	113 ± 98	<6	>×20
CD167	50 ± 5	6.5	×8
CD168	348 ± 122	ND[b]	ND[b]
PM274	570 ± 130	15.5	×37

[a]Primary skin fibroblasts were retrovirally transduced as described in *Materials and Methods*. Tg10 MoAb concentration in culture supernatants of cells cultured in monolayers or embedded into lattices were compared under standardized conditions of culture. Values represent the mean of three individual experiments.
[b]ND, not determined.

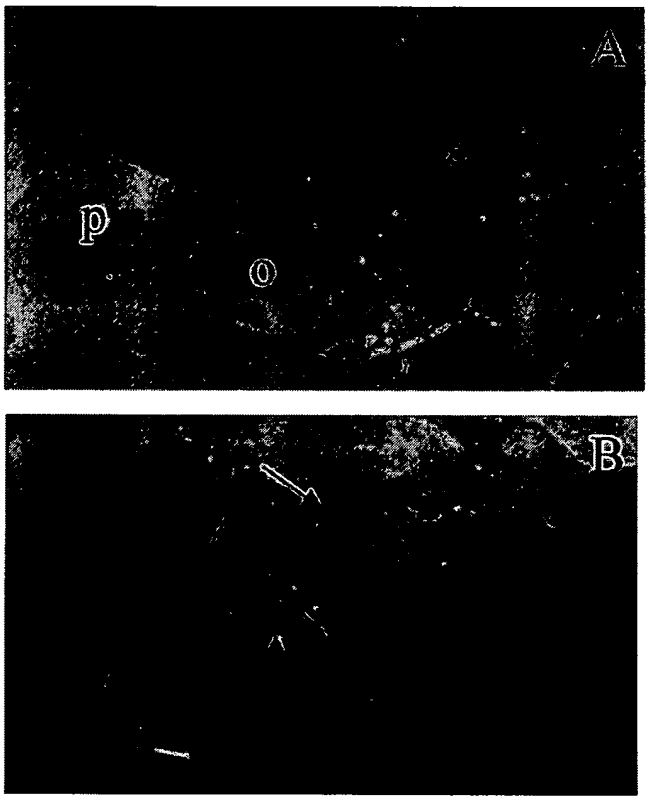

Figure 2. Skin fibroblast neo-organs. Lattices containing 10^7 cells each were formed *in vitro* and implanted intraperitoneally into adult C3H mice. Neo-organs were examined at various times postimplantation with no significant difference in their aspect. All neo-organs were found encapsulated into connective tissue and attached to the peritoneal cavity of the mouse. PTFE fibers can be seen in the inner part of the neo-organ (*arrow*). Vascularization is visible at the surface of the connective pouch and deeper into the neo-organ (*arrowhead*). o, organoid; p, peritoneum; g, gut.

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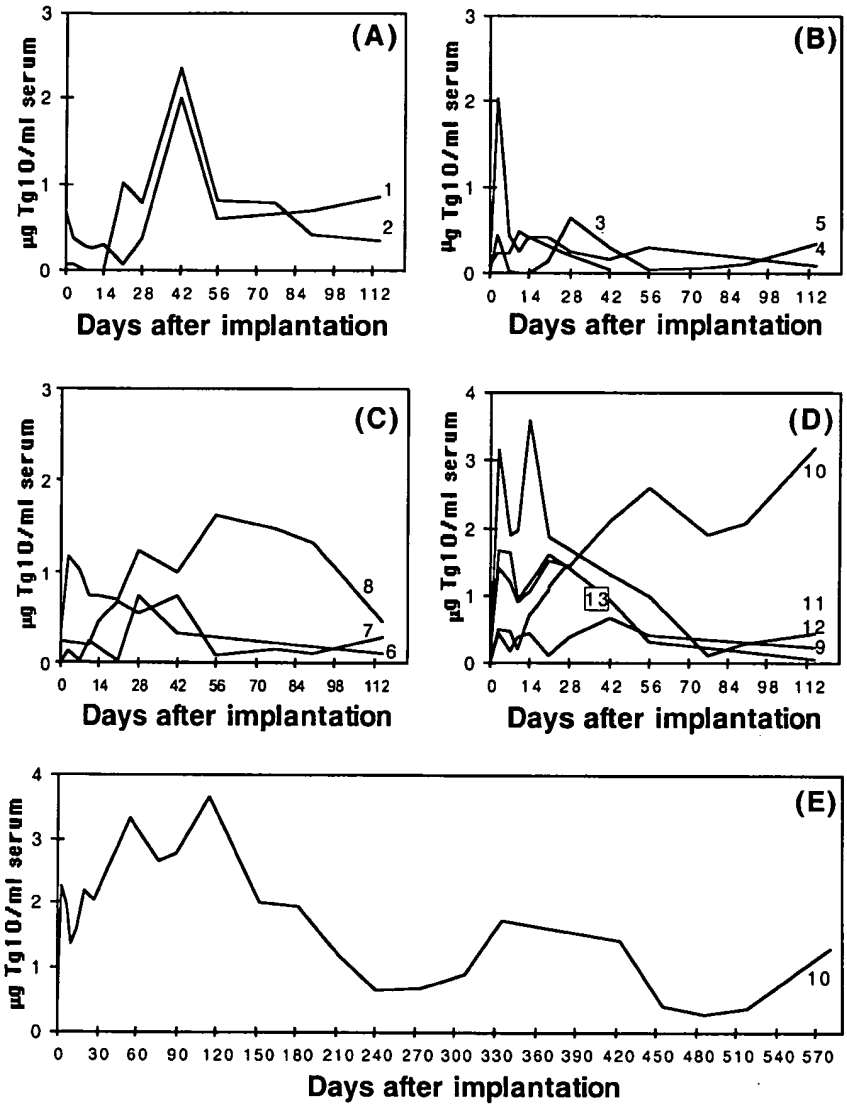

Figure 3. *In vivo* production of Tg10 MoAb by engineered skin fibroblasts. Two neo-organs containing 10^7 primary fibroblasts were implanted intraperitoneally into syngeneic mice. Blood samples were taken from animals at various time points and serum Tg10 MoAb was assayed by the ELISA. All animals were followed for 116 d (*A–D*) except one (mouse 10; *E*) that was followed for up to 19 mo. *A*, *B*, *C*, and *D* correspond to fibroblasts transduced by PM130, PM274, CD167, and CD168 vectors, respectively.

promoter, the ubiquitous PGK promoter. PM130 and PM274 were constructed in a MoMuLV backbone (vector pLXPXSN; Morgan *et al*, 1992) whereas CD167 and CD168 were constructed in an Akv backbone (vectors ptvAkv-tRNA^{Pro} and ptvAkv-tRNA^{Gln-1}, respectively; Lund *et al*, 1993) that provides a LTR less subjected to transcriptional repression than that of MoMuLV in various cell lineages. Moreover, CD168 differs from CD167 at the level of the tRNA primer binding site (see *Materials and Methods*) and no longer contains a transcription repressor element operating on the upstream LTR (Lund *et al*, 1993). Virus-producing cells were derived from either PA317 or FlyA amphotropic packaging cell lines (see *Materials and Methods*).

As a first step to our investigations, it was necessary to demonstrate that the four retroviral vectors were capable of the transfer and expression of antibody genes into skin fibroblasts. Three independent infection experiments using skin fibroblasts originating from different mice were conducted. No Tg10 MoAb was detected in culture supernatants of cells infected with an empty control vector (pLXPXSN; **Table I**). In contrast, antibody productions ranging from 50 to 570 ng per 10^7 cells per 48 h were observed with antibody gene-carrying vectors, CD158 and PM274 giving the best results (**Table I**). The fraction of infected cells was not precisely assayed. It is, however, likely that the observed differences in antibody production simply reflected differences in titers of the various viral suspensions used (see *Materials and Methods*). In conclusion, although not optimized for extensive cell infection, these experiments indicate that the four constructed vectors can transfer and express Tg10 cDNA in skin fibroblasts, at least *in vitro*.

Tg10 MoAb secretion by skin fibroblasts *in vitro* Before implantation into host animals, genetically modified skin fibroblasts are embedded into the matrix of collagen-coated PTFE fibers and cultured for 4 d *ex vivo*, a time necessary to allow the lattice to condense and form a compact structure (see *Materials and Methods*; Moullier *et al*, 1993a). Tg10 MoAb production by skin fibroblasts grown under standard conditions was compared with that of the same cells after condensation of lattices. A dramatic reduction was observed in all cases, with a diminution factor ranging from 8 to >37 (**Table I**). Comparable experiments having not been conducted for other proteins ectopically expressed by skin fibroblasts, it is not clear whether this effect is specific to antibodies. It, more probably, reflects a reduction in metabolic activity of cells undergoing proliferation arrest (Naffakh *et al*, 1995) or a reduced release of proteins secreted by cells cultured in three dimensions to the culture supernatant.

***In vivo* production and systemic delivery of Tg10 MoAb in mice** For each vector, two lattices containing 10^7 cells each were implanted in the peritoneal cavity of two to five C3H mice. One mouse (mouse 13) was killed for macroscopic examination of implanted lattices on day 21, another (mouse 3) on day 42 and all the other mice but one (see below) on day 116. All lattices formed

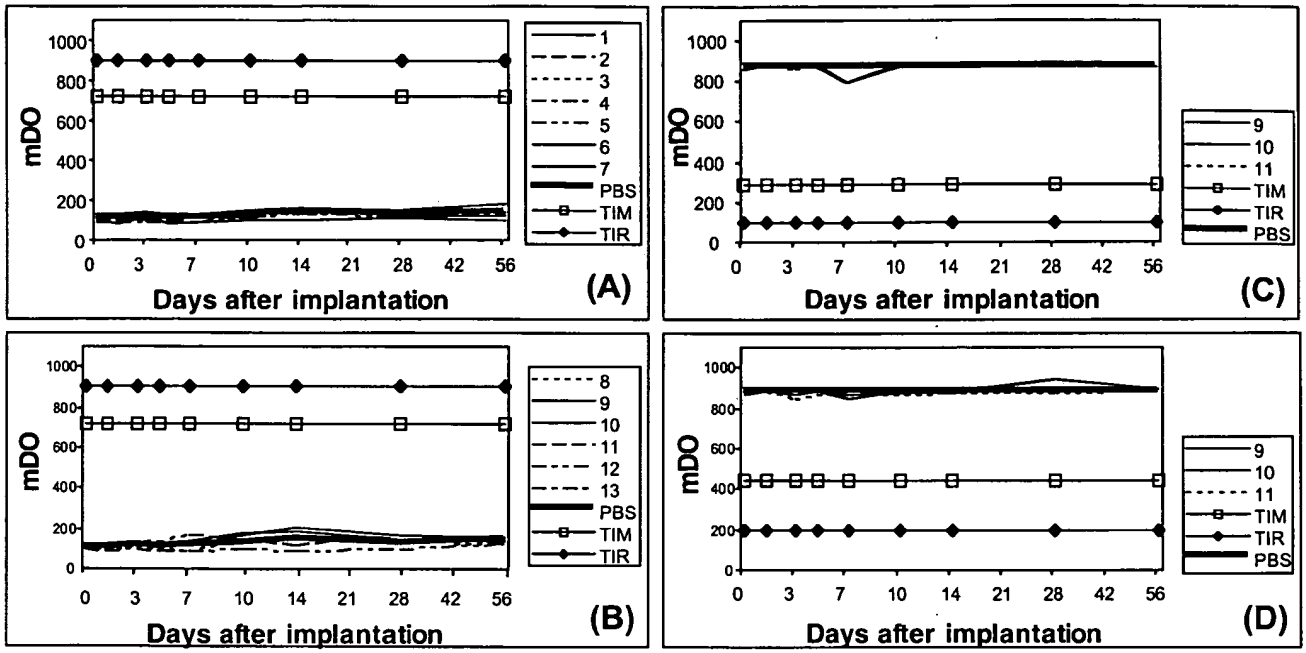

Figure 4. Analysis of the anti-idiotypic response against the ectopically expressed Tg10 MoAb. (*A*, *B*) The possible presence of anti-Tg10 antibodies in the serum (used at a 1/50 dilution) of all skin fibroblast-grafted mice (see **Fig 3**) was first assessed by direct ELISA as described in *Materials and Methods*. A serum (1/125 dilution) from a mouse immunized with purified Tg10 MoAb (TIM; Pelegrin *et al*, 1998b) and purified rabbit anti-Tg10 idiotype immunoglobulins (TIR: 10 μg per ml) (Del Rio *et al*, 1995) were used as positive controls. Phosphate-buffered saline was used as a negative control. (*C*, *D*) Sera from mice 9, 10, and 11, which gave slight positive signals in the direct ELISA (see **Fig 3*B***), were then analyzed in a competition (*C*) and an inhibition (*D*) ELISA as described in *Materials and Methods* using the TIM mouse and TIR rabbit positive controls as in *A* and *B*, and phosphate-buffered saline as a negative one.

neo-organs homogeneous in size, shape and aspect with no striking differences between days 21, 42, and 116. Neo-organs appeared as vascularized structures surrounded by a pouch of connective tissue and tightly associated with the peritoneum membrane over all their surface (**Fig 2**). With respect to this last point, they differed from those described by Moullier *et al* (1993b) and Naffakh *et al* (1995), which essentially appeared as stalked structures connected to either the peritoneum or bowel loops. It is, however, likely that this simply reflects a slight difference in the site of implantation of lattices. Importantly, and in keeping with observations by others (Moullier *et al*, 1993a,b), no trace of inflammation or necrosis was detected in any of the neo-organs examined.

Tg10 MoAb levels in the bloodstream were monitored by the ELISA at various time points (**Fig 3**). The data can be summarized as follows: (i) at least a transient Tg10 MoAb expression was detected in all treated animals; (ii) treated animals behave very heterogeneously with respect to Tg10 MoAb production; (iii) Tg10 MoAb production became undetectable on and after days 50–70 in half of the mice, whereas it was still detected in the other half on day 116; (iv) Tg10 MoAb can reach concentrations as high as 2–3 μg per ml in certain animals—however, variations of great amplitudes were observed for most animals during the time-course of the experiment; and (v) no retroviral vector gave results strikingly better than the others. With respect to the last point, it has been reported that the expression of genes transduced by MoMuLV-derived retroviral vectors is turned off *in vivo*, especially in fibroblasts, when driven by the LTR (Palmer *et al*, 1991), but not when driven by internal promoters such as those of dihydrofolate reductase or PGK genes (Scharfmann *et al*, 1991). In contrast with these findings, long-term secretion of the Tg10 MoAb was achieved here using both types of transcriptional control. Taken with observations by others that secretion of β-glucuronidase was possible for 5 mo upon transplantation of MPS VII mouse fibroblasts transduced with a LTR-driven retroviral vector (Taylor and Wolfe, 1997), this indicates that MuLV inactivation does not necessarily occur *in vivo* in engineered somatic cells.

Because Tg10 MoAb production was particularly high and stable in one mouse (mouse 10), the latter was not killed on day 116 and the follow-up for another 15 mo indicated no decline in antibody production (**Fig 3*E***).

No detectable anti-idiotypic response against the Tg10 MoAb in treated animals Injection in the presence of Freund's adjuvant has already shown that mice are competent for the development of anti-idiotypic responses against the Tg10 MoAb when appropriately immunized (Del Rio *et al*, 1995; Pelegrin *et al*, 1998a). It was thus important to rule out the possibility that engineered skin fibroblasts could play an adjuvant part favoring the initiation of an anti-idiotypic humoral response against the Tg10 MoAb. Previously characterized rabbit (Del Rio *et al*, 1995) and mouse (Pelegrin *et al*, 1998a) anti-sera containing anti-Tg10 MoAb antibodies were used as positive controls, and sera from mice subjected to no specific treatment were taken as being negative. In the first series of experiments, a direct ELISA aiming at detecting anti-Tg10 antibodies in the sera of skin fibroblast-grafted mice was used. Most animals proved negative except three (mice 9, 10, 11) that gave very low positive signals (**Fig 4*A*,*B***). Competition and inhibition ELISA, conducted as described in *Materials and Methods*, subsequently showed that these signals were non-specific (**Fig 4*C*,*D***), indicating that no detectable anti-idiotypic response developed in animals grafted with skin fibroblasts.

In conclusion, this study shows that primary skin fibroblasts are cells suitable for *in vivo* production and systemic long-term delivery of MoAb in immunocompetent animals. Although variations in the length and amount of antibody production were observed among the animals involved in this study, stable production of the Tg10 MoAb was observed for at least 19 mo in one animal. To our knowledge, this represents the longest time reported for the *in vivo* secretion of an ectopic protein by mouse skin fibroblasts. This indicates that ectopic antibody production by skin fibroblasts can be obtained for virtually the whole life-time of a mouse. Whether this conclusion can be extrapolated to humans, however, requires further

investigation. Variations in antibody production as well as loss of production in some mice were observed. Although it is not possible to exclude a selective elimination of antibody-producing cells from organoids (see below) at this stage of investigation, the examination of organoids in killed animals revealed no obvious difference in size and aspect. It must thus be taken into consideration that the decline in antibody production in most animals might be due to the fact that none of the vectors used in this study are optimal for long-term and sustained expression *in vivo* and that the development of efficient gene transfer/expression vectors is most likely a priority for long-term skin fibroblast-based gene therapy.

A possible limitation to gene/cell therapies aiming at the systemic delivery of exogenous proteins is the mounting of a treatment-neutralizing immune response (Isaacs 1990; Kuus-Reichel *et al*, 1994). The response can be: (i) humoral and essentially directed against the therapeutic protein where the latter is recognized as foreign, and/or (ii) cellular and directed to the genetically modified cells where the latter produce and present antigenic peptides derived from the ectopic protein. Interestingly, none of the animals receiving engineered skin fibroblasts showed a detectable anti-Tg10 idiotype antibody response in the experiments conducted. Similar results were obtained when the Tg10 MoAb was released from engineered primary myoblasts grafted into the tibialis anterior muscles of syngeneic mice (D.N., unpublished data). Taken together, these observations support the idea that non-plasmocytic cells are unlikely to play an adjuvant part favoring anti-idiotypic responses when engineered for antibody production. Also supporting the idea of the absence of any immune response against antibody-producing fibroblasts, regular examination of organoids indicated neither cellular infiltration, inflammation, or necrosis. This observation is in line with studies by Moullier *et al* (1993a,b) and Naffakh *et al* (1995) in experiments aimed at the production of other proteins. The development of sensitive cytotoxic T cell assays adapted to the study of skin fibroblasts, however, will be necessary to rule out formally and definitively the possibility that the loss of Tg10 MoAb expression might be accounted for by a selective cytotoxic T cell response against antibody-producing cells.

Finally, it is worth stressing that the grafting of cell-carrying PTFE lattices is not the only possible means for implanting genetically modified fibroblasts. Human fibroblasts encapsulated in Baxter theralyte devices have, for instance, been used successfully for the production of human growth hormone for more than 1 y in mice (Josephs *et al*, 1999) and we have recently shown that systemic delivery of MoAb by cells encapsulated in cellulose sulfate is particularly efficient without inducing any anti-idiotypic response (Pelegrin *et al*, 1998a). Cellulose sulfate is a material certified for human use and presents a number of advantages over other encapsulation devices (Dautzenberg *et al*, 1999). It will, thus, be interesting to test whether cellulose sulfate-encapsulated engineered skin fibroblasts can survive and produce antibodies in the long term. Should this prove to be the case, this approach would also allow the extension of the use of such cells in allogenic implantation by exploiting the immunoprotective properties of cellulose sulfate capsules (Dautzenberg *et al*, 1999).

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